

PLATELET ASSOCIATED IMMUNOGLOBULIN ASSAY KIT

This kit is designed for the quantitation of platelet associated immunoglobulin using a flow cytometer. In addition, the kit provides the capacity to identify the class (IgG, IgA or IgM) of immunoglobulin associated with the platelets.

Each kit contains reagents sufficient for 25 tests.

CATALOG NO: ABK650 (Silenus number: 989060995)

SUMMARY: Tests for elevated levels of platelet associated immunoglobulin (PAIg) are requested frequently in cases of thrombocytopenia of unknown aetiology. This kit introduces rapid and reliable measurement and identification of class (IgG, IgA or IgM) of platelet associated immunoglobulin using a flow cytometer.

PRINCIPLE: For the investigation of PAIg, flow cytometry has the advantage over other techniques e.g., radioimmunoassay, complement fixation or protein. A binding, since it can be performed on specimens with low platelet counts ($<5 \times 10^9/l$). In addition, it is not necessary to have a pure platelet preparation. The kit incorporates an R phycoerythrin (R-PE*) conjugated monoclonal antibody to platelet glycoprotein IIb (CD41) to assist with gating, ensuring selection of the platelets for evaluation. Although platelets can be selected by simple forward angle and side (90°) scatter gating, an overlap may occur with debris and other non platelet events. This is most noticeable when the patient is severely thrombocytopenic and collection of a large numbers of events is required in order to achieve a statistically viable number of platelets for evaluation. In such cases the dual staining approach offered by this kit will be useful as, without the R-PE* conjugated gating reagent it would not be possible to identify the platelets among the large amount of debris and other non platelet events that would inevitably accompany them in the acquired data set. Tests designed to quantify IgG only are ill advised, as it is known that elevated levels of immunoglobulins of other classes (IgA and IgM) are found in association with platelets in a significant number of cases of immune thrombocytopenic purpura (ITP). The kit provides five fluorescein isothiocyanate (FITC) conjugated polyclonal antibodies as follows: An antibody which is polyspecific for total human immunoglobulins, reagents which are monospecific for immunoglobulins of classes IgG, IgA and IgM and an anti rabbit immunoglobulin as a negative control.

STORAGE AND STABILITY: To ensure stability of the reagents until the stated expiry date, store the kit at 2 to 8°C except when in use (do not freeze).

MATERIALS PROVIDED:	Gating reagent: CD41 R-PE* conjugated monoclonal antibody to platelet glycoprotein IIb	1 bottle (1.25 mL)
	FITC conjugated polyclonal antibody to total human immunoglobulins	1 bottle (0.25 mL)
	FITC conjugated polyclonal antibody to human IgG	1 bottle (0.25 mL)
	FITC conjugated polyclonal antibody to human IgA	1 bottle (0.25 mL)
	FITC conjugated polyclonal antibody to human IgM	1 bottle (0.25 mL)
	FITC conjugated polyclonal antibody to total rabbit immunoglobulins	1 bottle (0.25 mL)

**MATERIALS REQUIRED
BUT NOT PROVIDED:**

Blood collection tubes containing EDTA as anticoagulant.

Test tubes compatible with the sampling system of the flow cytometer to be used.

Micropipettors. Transfer pipettes.

Vortex mixer.

Benchtop centrifuge capable of 700g.

Wash buffer (see reagent preparation).

Flow cytometer equipped with a laser capable of exciting R-PE* and FITC - 488nm wavelength, and a filter configuration suitable for detection of the emitted signals.

REAGENT PREPARATION:

Wash Buffer: Phosphate buffered saline, containing 10mM EDTA and 0.5% bovine serum albumin (PBS/EDTA/BAS) is recommended for washing and resuspension of the platelets.

Kit Reagents: These are ready to use and no further preparation is required.

PROCEDURE:

1. Collect venous blood into EDTA anticoagulant. 3 to 5 mL of blood will be sufficient in most cases and should be processed within 4 hours.
2. Centrifuge blood at low speed (approx. 100g) for 15 minutes to obtain platelet rich plasma.
3. Centrifuge the platelet rich plasma at approx. 700g for 2.5 minutes, aspirate and discard the plasma, replacing it with an equal volume of wash buffer (PBS/EDTA/BSA). Resuspend the platelets by agitation using a vortex mixer if necessary.
4. Wash the platelets 3 times by centrifuging at 700g for 2.5 minutes, aspirating the supernatant and resuspending the platelet pellet in fresh wash buffer.
5. Resuspend the wash platelets at 1 to 5×10^9 /mL (this concentration is given as a guide to avoid antigen excess in the assay and may not be achievable in cases of severe thrombocytopenia).
6. Label 5 flow cytometer tubes, one each for the negative control, total immunoglobulins, IgG, 1gA and IgM.
7. Place 10 μ l of the gating reagent, CD41 R-PE* into each tube.
8. Add 10 μ l each of the remaining five reagents to the appropriately labelled tubes.
9. Pipette 100 μ l of the platelet suspension into each tube, mix gently and incubate, in the dark, at room temperature for 15 minutes.
10. Wash the platelets twice as described in step 4.
11. Resuspend the platelets in approximately 0.5 mL PBS/EDTA/BSA and analyse using a flow cytometer with forward angle light scatter and side (90°) scatter parameters set to logarithmic amplification.
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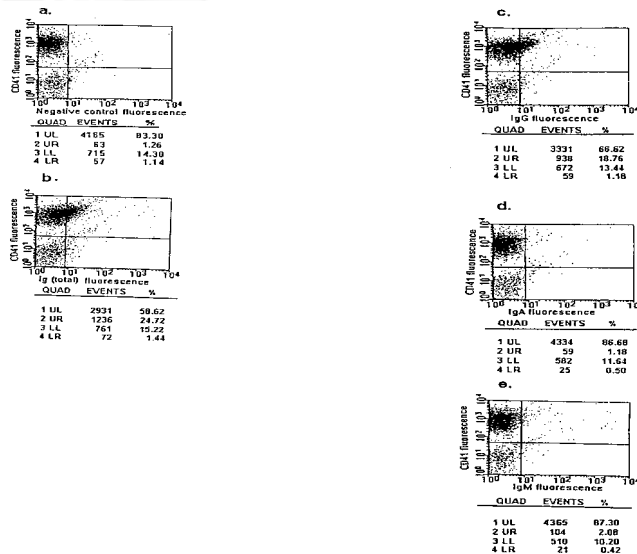
DATA ANALYSIS:

The use of quadrant statistics corrected, for non platelet events is recommended as follows :

- (i) Display the negative control fluorescence data, ungated, as a log scale dot plot of FITC fluorescence (signal derived from anti rabbit Ig) versus R-PE* fluorescence (signal derived from CD41 antibody).
- (ii) Set quadrants to define negative for both fluorescence parameters on the basis of the distribution of events acquired from the negative control, as shown in Figure 1a.
- (iii) To determine the background stained percentage, express dual stained events (upper right quadrant) as a percentage of total CD41 positive events (upper left + upper right); typically this corrected value will be <2%.
- (iv) Keeping the quadrants fixed in this position, display the corresponding data acquired for the remaining tubes, in which CD41 was combined, respectively, with polyclonal antibodies to total immunoglobulins, IgG IgA and IgM as shown in Figure 1 b to e.
- (v) For each data set, repeat the calculation to obtain corrected values for percentages of dual stained events.
- (vi) Finally, subtract the background stained percentage from the corrected values obtained for total immunoglobulins, IgG, IgA and IgM.

FIGURE 1

Example of data from a patient with a marginally elevated IgG class PAIg analysed using dual fluorescence dot plots and quadrant statistics corrected for non platelet events. The corrected percentage of dual stained events for the negative control (a) is 1.5%. This value is subtracted from the corrected percentages calculated for the remaining dot plots to give the following results: (b) Ig (total) 28.2%, (c) IgG 20.5%, (d) IgA 0%, (e) IgM 0.8%.



INTERPRETATION OF RESULTS:

Inhouse reference ranges must be established by the laboratory performing the tests.

**WORKING WITH LOW
PLATELET NUMBERS:**

The difficulties of measuring PAIg in the face of low platelet counts can be overcome, largely, by dual colour flow cytometry. In cases of severe thrombocytopenia, however, it may be necessary to acquire a large number of events (50,000 or more) in order to achieve a statistically viable number of platelets for evaluation.

**PERFORMANCE
LIMITATIONS:**

False positive results may be obtained if long periods of time elapse between drawing the blood sample and performing the test. The test should be performed within four hours of drawing the blood.

**WARNINGS AND
PRECAUTIONS:**

Test specimens are potentially infectious and should be handled with care. The kit reagents contain sodium azide as a preservative. Unwanted reagent should be disposed of with liberal volumes of water to prevent the formation of potentially explosive metallic azides in metal plumbing.

**SUGGESTED FURTHER
READING:**

Ault KA. Flow cytometric measurement of platelet associated immunoglobulin. *Pathol Immunopathol Res* 1988, **7**:395-408.

Borne A E G KR von dem et al. Clinical significance of positive platelet immunofluorescence test in thrombocytopenia. *Br. J Haematol* 1986, **64**:767-776.

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McMillan R. Clinical role of antiplatelet antibody assays. *Seminars in Thrombosis and Hemostasis*. 1995, **21**:37-45.

Rosenfeld CS et al. Flow cytometric measurement of antiplatelet antibodies. *Am J Clin Pathol* 1987, **87**:518-522.

***Patents : US; 4,520,110, Europe; 76,695, Canada; 1,179,942, Australia; 548,440**

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